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SEPARATION OF ALKALINE PHOSPHATASE ISOENZYMES FROM VARIOUS RAT --ETC(U)

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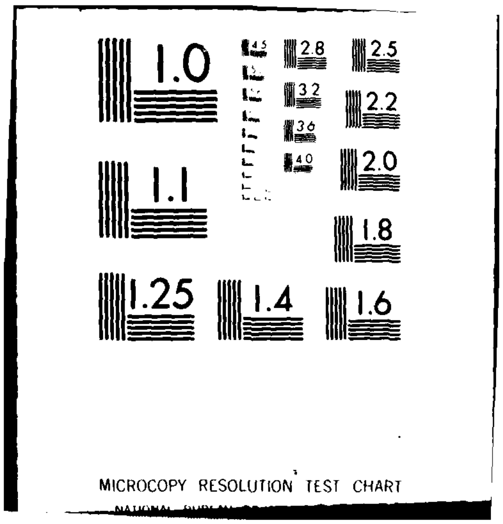
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Relative alkaline phosphatase (AP) activity and isoelectric point (pI) values of AP isoenzymes were determined for a variety of rat tissues. Prepared tissue samples were placed on polyacrylamide gel and were subjected to isoelectric focusing (IEF). The gel was stained for AP and the intensity of staining was found to relate to the measured biochemical AP activity.

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SEPARATION OF ALKALINE PHOSPHATASE ISOENZYMES FROM VARIOUS RAT TISSUES USING FLAT-BED ACRYLAMIDE GEL ISOELECTRIC FOCUSING

SYNOPSIS

Relative alkaline phosphatase (AP) activity and isoelectric point (pI) values of AP isoenzymes were determined for a variety of rat tissues. Prepared tissue samples were placed on polyacrylamide gel and were subjected to isoelectric focusing (IEF). The gel was stained for AP and the intensity of staining was found to relate to the measured biochemical AP activity.

INTRODUCTION

Alkaline phosphatase represents a class of lysosomal hydrolases present in many types of mammalian tissues. Specific isoenzymes of alkaline phosphatase have been identified as originating from active osteoblasts, growing connective tissue, and tissue in which transcellular ion transport is an important function.[1,2]

Each different isoenzyme of alkaline phosphatase has a characteristic electrophoretic mobility that can be modified when the alkaline phosphatase is first treated with neuraminidase before being placed on a polyacrylamide gel.[3-8] An isoenzyme's characteristic electrophoretic mobility can be visualized on a polyacrylamide gel as a specific band. The specific band that is developed is indicative of a particular alkaline phosphatase isoenzyme. Therefore, the identification of isoenzymes from different tissues can be accomplished.

Isoelectric focusing (IEF) employing a polyacrylamide gel has been used for the investigation and identification of alkaline phosphatase isoenzyme bands.[7,9,10] In the technique of IEF, a particular isoenzyme will demonstrate a characteristic isoelectric point (pI) (because of its electrophoretic mobility) that can be seen as a band on the polyacrylamide gel. IEF is directly applicable to studies where isoenzymes of alkaline phosphatase must be identified, such as in osseous wound repair.

The technique of IEF is a simple laboratory procedure that can

produce predictable, reproducible, and accurate results. Unfortunately, there is a paucity of information in the scientific literature to provide the investigator with the pI values for alkaline phosphatase isoenzymes of experimental animals. It is the purpose of this paper, therefore, to gather pI values of alkaline phosphatase isoenzymes and evaluate relative alkaline phosphatase activity from a variety of rat tissues. These values can be used as the standards with which future data may be compared.

MATERIALS AND METHODS

Serum and tissue alkaline phosphatases were prepared from 15 female Walter Reed strain of rats. The rats were anesthetized using sodium pentabarbital (3-5 mg/100 mg body wt) and blood was withdrawn by cardiac puncture. The blood was allowed to clot for 30 minutes at room temperature and was spun on a centrifuge for 10 minutes. The serum was recovered and was stored at -20°C until it was needed.

Animals were sacrificed with an overdose of sodium pentabarbital and the liver and kidney were surgically removed. These organs were prepared for IEF using a modification of the method of Righetti and Kaplan.[3] The same organs were grouped together and placed into cold 0.25M sucrose and were minced with scissors and homogenized in a Potter-Elvehjem tissue grinder at 3C in 5 vols of 0.25M sucrose. The homogenate for each group was prepared for extraction by the addition of cold N-butanol over a 15 minute time period to a volume of 20% (v/v). The extraction involved 30 minutes of continual stirring.

The product was spun in a centrifuge at 38,000 g for 30 minutes and the aqueous layer was removed and stored at -20°C.

Endochondral (tibia) and intramembranous (parietal) bones were surgically removed, placed into cold 0.25M sucrose and were separately grouped according to genesis. The bones were scraped free of any adhering soft connective tissue and were smashed in a precooled mortar and pestle. The crushed samples were put in a sonicator in 4 vols of 0.25M sucrose at 60% pulse for 3 minutes at 5°C on a Branson cell disruptor. The liquid phase (supernatant) was removed and placed into a centrifuge and spun at 5000 g at 5°C for 10 minutes. The liquid phase from this procedure was prepared for extraction in the manner described earlier for the soft tissue specimens.

I. Enzyme Activity and Total Protein

Alkaline phosphatase activity was determined by using p-nitrophenylphosphate as the substrate. The determination was run at 37°C at a pH of 10.3 using a Beckman DU-2 spectrophotometer. The assay method employed was adapted from a Sigma Company Technical Bulletin No. 104. Alkaline phosphatase activity was expressed as 1 micromole of p-nitrophenol hydrolyzed per hour and specific alkaline phosphatase activity was defined as the number of micromoles of p-nitrophenol hydrolyzed per hour per microgram of protein.

The total protein was determined according to a procedure described in Technical Bulletin No. 1051 from BIORAD Laboratories.

II. Isoelectric Focusing (IEF)

Extraction mixtures were run on polyacrylamide flatbed gels purchased from LKB Instruments. The gels were run at a pH range of 3.5 to 9.5 for 1.5 hours. The conditions established for the runs were as follows: power - 25 W; current - 50 mA; and voltage - 1500 V. The bed was maintained continually at 5°C. The samples (soft and hard tissues and serum) were applied to the bed using wicks containing 10-15 microliters of the tissue extract. Congo red and Evans blue were used as internal standards.

Flat-bed gels were stained for alkaline phosphatase by a modification of the method of Kaplan and Rogers.[11] Isoenzyme bands were developed by placing the gel in 1M Tris-maleate solution of pH 9.8 with 3.0 mM magnesium sulfate for 10 minutes. The gel was then transferred to a solution of 0.25 M Tris-maleate, pH 9.8, with 0.8 mM sodium alpha-naphthyl phosphate for 45 minutes. Next, the gel was put into distilled water for 10 minutes and then into a solution of 0.25 M Tris-maleate, pH 9.8 with 4-aminodiphenylamine diazonium dye (100 mg per 200 ml). The isoenzymes appeared as brown bands on the gel and they were scanned with a densitometer.*

RESULTS

I. Alkaline Phosphatase

The alkaline phosphatase activity and the specific enzyme

*Zeineh Soft Laser Scanning Densitometer, Biomed Instruments, Inc. Marketed by LKB Instruments, Inc., Rockville, MD 20852

activity of the tissues were determined both before and after extraction procedures. The values obtained may be seen in Table 1.

II. Isoelectric Focusing: pI Values

The serum samples always showed three principal isoenzyme bands (pI values of 4.35, 4.45, and 5.85) that were present on every gel that was run. The fastest moving band was slightly more intense in color than the slower moving bands. In several runs the serum also displayed secondary bands (with different pI values) in addition to the three principal bands. The infrequently occurring bands were less intense in color than the principal bands and appeared between the 2nd and 3rd principal bands.

The kidney extracts consistently showed eight distinct isoenzyme bands. The two most intensely staining bands were also the fastest moving bands. The pI of these two bands were 4.35 and 4.45. The remaining six bands decreased in intensity towards the cathode. The pI of these bands was grouped according to similar intensity. Therefore, the isoelectric points in terms of this grouping were 5.6 and 5.3; 6.0 and 5.8; and lastly, 6.25 and 6.55.

The liver extract yielded inconclusive results. The bands that were produced stained lightly, and did not consistently appear in the runs. Therefore, it is not prudent to make any definite statements concerning the pI of liver isoenzymes, at this time, using the technique described.

The bone extracts of isoenzymes from endochondral and intramembranous sources displayed either one distinct and intensely

staining band at a pI of 4.45 or a diffusely staining and broad band with a pI ranging from 4.9 to 4.45. Bands from endochondral and intramembranous sources were the same for all the gels that were run.

In terms of electrophorytic mobility, the most rapidly moving bands on the flat-bed acrylamide gel were those developed from the alkaline phosphatase isoenzymes of bone and kidney at a pI of 4.45. The fastest band for serum was at a pI of 4.35. A band that was developed at an isoelectric point (pI) of 5.85 was commonly shared by the slowest moving isoenzyme from serum and one of the isoenzymes of the kidney. The results of the IEF may be seen in Table 2.

DISCUSSION

The serum AP from the rat showed three primary bands with unique pI values. Similar primary bands and pI values from human serum suggest a possible relationship between certain alkaline phosphatase isoenzymes from different mammalian species, indicating that these substances probably have similar stearic conformations.

In the rat, a singular, common alkaline phosphatase isoenzyme band was identified from both the femur and parietal bone extracts and it showed a pI of 4.45. The actual AP activity for the femur, however, was much greater than for the parietal bone: 108.17 μmol/hour and 23.83 μmol/hour, respectively. The difference may be associated with the endochondral genesis of the femur in contrast to the intramembranous origin of the parietal bone. However, we may conjecture that because the long bones (i.e., femur) are constantly

stressed during daily use, osteoblastic maintenance activities must be performed to insure for structural integrity. Osteoblast performance is closely associated with alkaline phosphatase, therefore, it is not unexpected to find higher activity levels of this enzyme in a femur as compared with a nonstressed, sedentary flat bone of the cranium (i.e., parietal).

Using IEF, the rat kidney displayed eight major bands indicative of AP. A study by Nose[10] of rat kidney AP demonstrated two major isoenzymes and several minor isoenzymes. It is possible that the difference in the number of bands and pI values may be a consequence of a variation in the amino acid sequence that is characteristic for each alkaline phosphatase isoenzyme.

The values of AP for the kidney were high relative to other tissues that were examined. This was not an unexpected finding because there is an abundance of alkaline phosphatase located at the brush border of the epithelial cells of the proximal tubules.

The liver pI values could not be precisely determined. Liver alkaline phosphatase bands were infrequently developed, and it was not possible to determine their pI values because the bands were diffuse and lightly staining. Liver alkaline phosphatase is labile, therefore, it is difficult to identify after extraction. A future study is anticipated to thoroughly evaluate both kidney and liver alkaline phosphatase isoenzymes.

CONCLUSION

This study has shown that alkaline phosphatase isoenzymes can be effectively and easily separated using flat-bed acrylamide gel isoelectric focusing. However, the technique for liver enzyme separation and characterization must be modified.

The technique described can be readily used to evaluate a wide variety of tissue where alkaline phosphatase activity is known or suspected. Importantly, the procedure is easily adapted for acid phosphatase examination by merely changing the pH and the substrate.

* * * *

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the U. S. Army Medical Department.

TABLE 1. Values of alkaline phosphatase activity for homogenates and extracts of different tissues.

	HOMEGENATE		EXTRACT	
	$A.P. \pm \overline{S.D.}_{A.P.}$*	S.P. ACT†	$A.P. \pm \overline{S.D.}_{A.P.}$	S.P. ACT
Serum	5.54±0.71	0.12±0.01	19.45±10.76	0.48±0.21
Liver	‡	‡	1.82±0.81	0.21±0.14
Kidney	913.5±105.13	49.25±8.82	405.80±76.85	82.71±29.92
Parietal Bone	33.07±8.06	52.55±9.01	23.83±3.73	432.0±81.69
Femur	197.2±11.03	29.84±15.25	108.17±25.97	188.0±62.60

*$A.P. \pm \overline{S.D.}_{A.P.}$ = Alkaline phosphatase activity in µmol/hr ± mean standard deviation.

†S.P. ACT = Specific alkaline phosphatase activity in µmol/hr/mg protein.

‡Due to labile nature of the liver alkaline phosphatase, activities could not be determined from homogenates.

TABLE 2. pI Values of Pooled Samples.

TISSUES	pI Values							
Sera	4.35	4.45						
Kidneys		4.45	4.85	5.30	5.60	5.85	6.00	6.25	6.55
Femurs		4.45	4.45*					
Parietal Bones		4.45	4.45*					

*= a diffuse band from 4.45 to 4.90

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